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for Breast Cancer Chemotherapy

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14. ABSTRACT Cell membrane-associated and intracellular drug resistance mechanisms are the major cause of breast cancer treatment failure. The aim of this proposal is to develop nuclear localizing nanoparticles to delivery DNA-toxins breast cancer cell nuclei to effectively overcoming the drug resistance. We finished the task 1 - To synthesize and optimize folic-acid- or LHRH-functionalized charge reversal nanoparticles. The cationic polymer PEI and its block copolymer with degradable PCL were synthesized. The cationic PEI block amines were converted to acid labile amides to obtain the PCL-PEI/amide block copolymer. This polymer was used to fabricate the nanoparticles. The ideal nanoparticles with optimal sizes and acid-triggered negative-to-positive charge reversal properties were fabricated and characterized carefully. These nanoparticles will be characterized in vitro and in vivo.					
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Introduction

We worked according to the TASK 1 in the SOW:

STATEMENT OF WORK

Breast Cancer-Targeted Nuclear Drug Delivery Overcoming Drug Resistance for Breast Cancer Chemotherapy
University of Wyoming, 1000 E Univ Ave, Laramie, Wyoming

Maciej Radosz (PI)
Youqing Shen, Ph.D. (Co-PI)
William J. Murdoch, Ph.D. (Co-PI)

TASK 1. To synthesize and optimize folic-acid– or LHRH-functionalized charge reversal nanoparticles (12 Months):

- a. Synthesize linear polyethyleneimine (PEI, Mn ~0.8-10kDa) by ring-opening polymerization.
- b. React the PEI with proper 5-membered ring-anhydrides to prepare charge-reversal PEIs (PEI/amides), characterize and optimize their charge-reversal kinetics.
- c. Introduce folic acid or LHRH to the PEI/amides using a post-reaction method.
- d. Fabricate and characterize TCRNs.
- e. Load drugs doxorubicin (DOX), camptothecin (CPT) and other drugs for breast cancer to TCRNs.

Milestone 1: To obtain the FA- and LHRH-functionalized TCRNs with optimal charge-reversal kinetics, targeting group density, size, and drug loading.

TASK 2. To in vitro evaluate the TCRNs for breast cancer chemotherapy (12 Months):

- a. In vitro test drug release profile at pH 7.4.
- b. Test stability in blood.
- c. In vitro test cellular binding (competitive inhibition method).
- d. In vitro test cellular uptake of TCRNs (flow cytometry, confocal laser-light scanning fluorescence microscopy).
- e. Intracellular trafficking.
- f. In vitro cytotoxicity to breast cancer cells.

Milestone 2: To screen out the TCRNs with the highest in vitro anti-breast cancer activity.

TASK 3. To in vivo evaluate TCRNs' anti-breast cancer efficacy (12 months)

- a. In vivo test biodistribution and tumor targeting efficiency using nude mice (about 120 mice).
- b. In vivo test and compare anticancer activity using nude mice with ip tumors and sc tumors treated by ip and iv injections (about 200 mice).

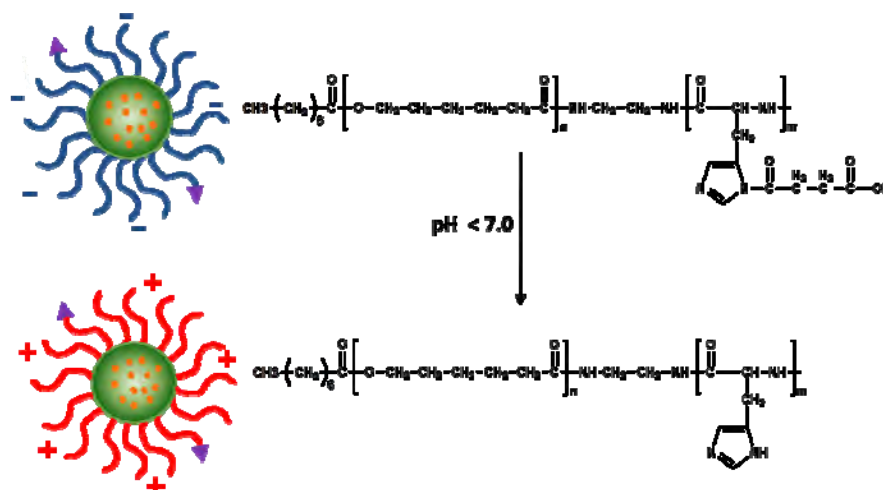
Milestone 3: To screen out the TCRNs with the highest in vivo anticancer activity

pH-Responsive Polyhistidine Nanoparticles for Nuclear Targeted Drug Delivery

1. Background

Last year we used cationic polymer poly(ethyleneimine) (PEI) as the charge-reversal polymers and demonstrated breast-cancer-targeted nuclear drug delivery carriers, but we found that the ability of the PEI to disrupt the endosome/lysosome membrane was not very effective. Furthermore, it is also not degradable. Polyhistidine (PolyHis) has strong fusogenic activity in addition to pH-sensitive ability and biodegradability. Its imidazole groups are only partially protonated at physiological pH but fully protonated at lysosomal/endosomal pH.¹⁻² The protonated imidazole groups can fuse with lipid bilayers by interacting with the negatively charged phospholipid membrane, efficiently disrupting endosomes/lysosomes.³ However, polyhistidine can only be used as hydrophobic blocks since it is water-insoluble at the neutral pH.³⁻⁵

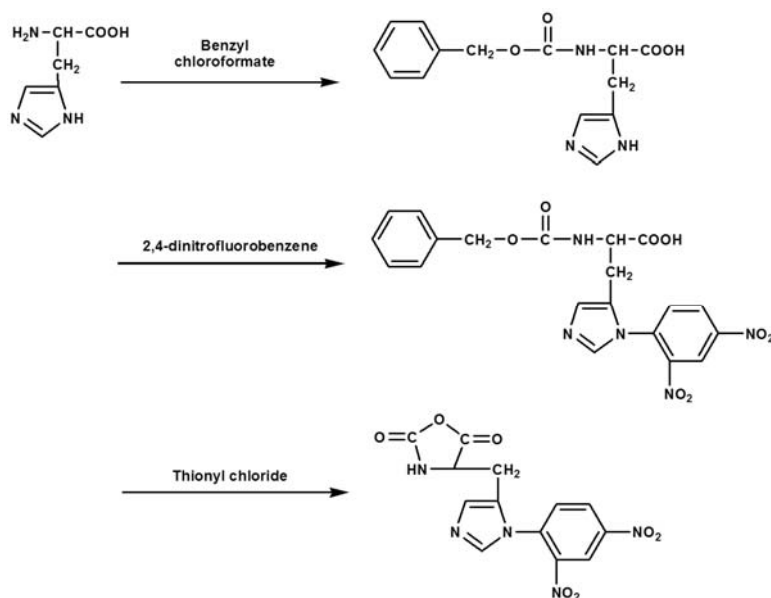
Herein, we amidized PolyHis (PolyHis^s) to make it water-soluble and negatively charged at the neutral pH, but PolyHis^s converted back to PolyHis having strong lysosomal lysis ability at lysosomal pH. Its block copolymer with PCL (PCL-PolyHis^s) formed nanoparticles with a diameter of 130 nm at the neutral pH. The nanoparticles were negatively charged at pH 7.4 because of the ionization of the succinamic acid groups. At lower pH (pH < 6.0), the imidazole amide of succinamic acid groups hydrolyzed quickly and the nanoparticles changed to positively charged because of the protonation of the regenerated imidazole groups and the lysosomal membrane disruption ability of PolyHis was thus regained.



Scheme 1. The amidized Polyhis nanoparticle and its pH-triggered charge reversal.

2. Results and discussion

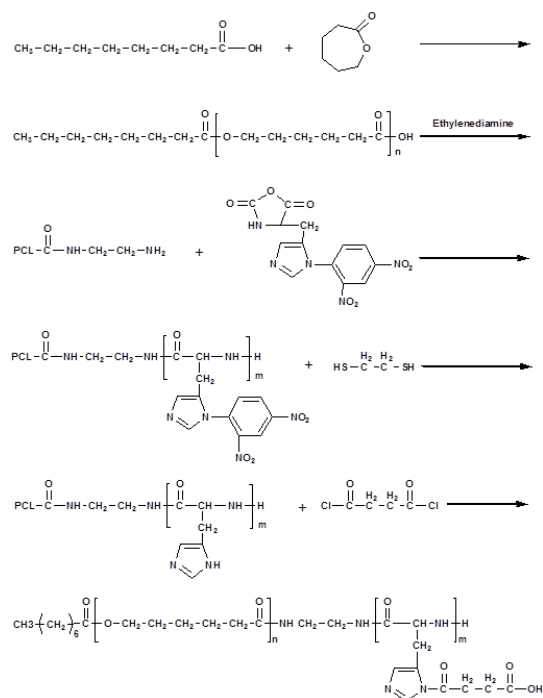
2.1. Synthesis of the pH-sensitive PCL-PolyHis^{-S} Nanoparticles



Scheme 2. Synthesis of the polyhistidine monomer.

The monomer was first synthesized according to the method reported by Bae et al.⁵ Polycaprolactone ($M_n = 2000$)-*block*-PolyHis^{-S} ($M_n = 5000$) (PCL-PolyHis^{-S}) copolymer was synthesized as shown in Scheme 3. PCL with terminal carboxylic acid group (PCL-COOH) was synthesized using octanoic acid as the initiator. The acid group was reacted with an excess of ethylenediamine to form PCL with terminal primary amine group (PCL-NH₂). It initiated the polymerization of the NCA monomer to produce PCL-*block*-poly(N^{im}-DNP-L-histidine). The DNP groups were removed with 2-mercaptoethanol to produce PCL-Polyhis. The structure of the block copolymer PCL (DP = 18)-b-Polyhis (DP = 23) was characterized by ¹H-NMR and GPC (Figure 1).

PCL-Polyhis reacted with an excess of succinyl chloride in DMF and amidized the imidazole amine into succinoamide, producing PCL-succino-amidized polyhis (PCL-Polyhis^{-S}). The product was purified by dialysis against pH 8.0 PBS.



Scheme 3. Synthesis of the PCL-block-polyhistidine and its amidized product PCL-Polyhis^{-S}.

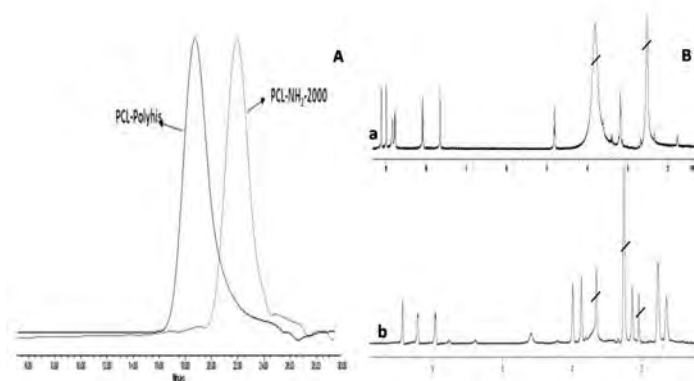


Figure 1. The GPC traces of PCL-NH₂ and PCL-*block*-Polyhis (A); The NMR spectra of the NCA (B, a) and the PCL-*block*-Polyhis (B, b).

2.2. Formation of Nanoparticles

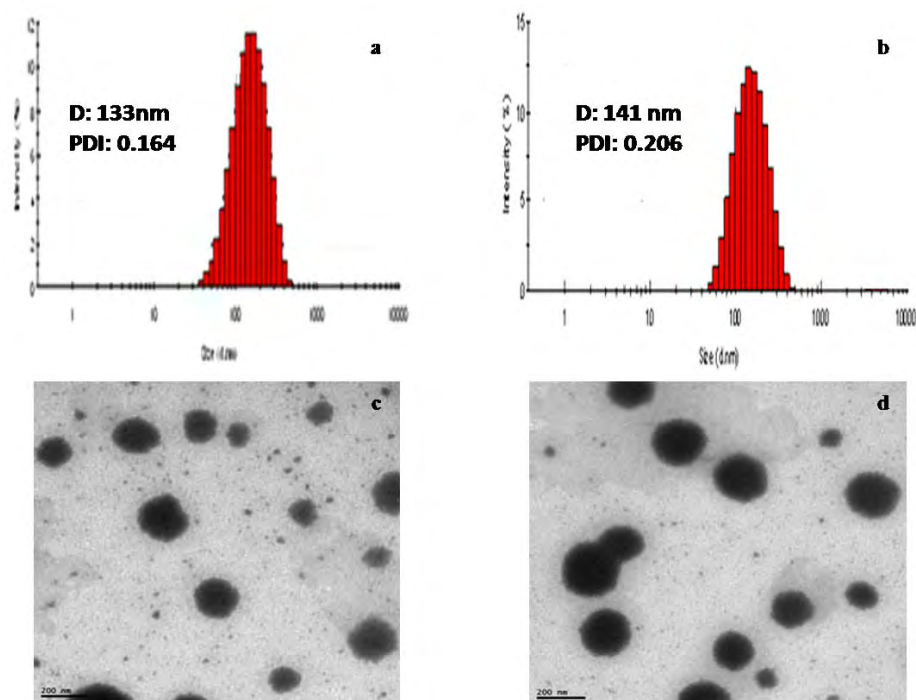


Figure 2. The size distribution of PCL-Polyhis^S nanoparticles (a) and PCL-Polyhis^S loaded with 12.9% DOX measured by dynamic laser light scattering (DLS) and the transmission electron microscopy (TEM) images of PCL-Polyhis^S nanoparticles (c) and PCL-Polyhis^S/DOX nanoparticles (d).

The size distributions of the PCL-Polyhis^S nanoparticles with or without DOX loaded are shown in Figure 2. The size of the PCL-Polyhis^S nanoparticles in PBS (pH 7.4) solution was about 133 nm in diameter and became slightly larger to 141 nm in diameter (Figure 2b and 2d) after loaded 12.9% DOX. Their spherical structures were confirmed by TEM. Their CMC and drug release kinetics are shown in Figure 3.

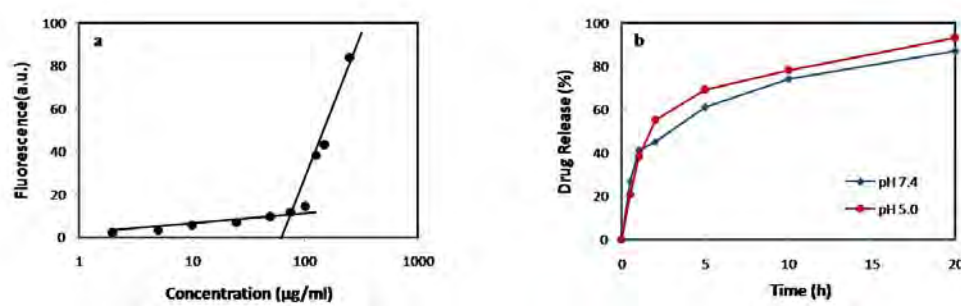


Figure 3. (a) The hydrolytic kinetics curves of the PCL-polyhis^S at different pH as a function of time detected by NMR. (b) The ζ -potential of the PCL-polyhis^S nanoparticles as a function of time at different pH.

2.3. The pH-triggered hydrolysis of the succinamic Acid

The first and most important role of the compound chosen to modify the Polyhis is to enhance the water solubility of the Polyhis greatly at neutral pH. Also, the group should have no hydrolysis at physiological pH

(pH 7.4), which would keep the stability of the nanoparticles, but should quickly hydrolyze to regenerate Polyhis at the tumor extracellular pH (pH < 7) or the endosomal/lysosomal pH (pH 4-5) for the endosomal/lysosomal escape to release the drugs around or even into the nucleus.

The stability of the β -carboxylic acid amide of imidazole in PCL-Polyhis^S was estimated by analysis of its hydrolysis at pH values of 7.4, 6.0 and 5.0 using NMR (Figure 4a). The amide very slowly hydrolyzed at pH 7.4, less than 5% in 24 h and only 7% in 48 h, while at weakly acidic pH, 6.0 or 5.0, the amide hydrolyzed quickly, 50% at pH 6 and 82% at pH 5 in 12 h. NMR results showed that the hydrolysis of the amide regenerated the imidazolyl group.

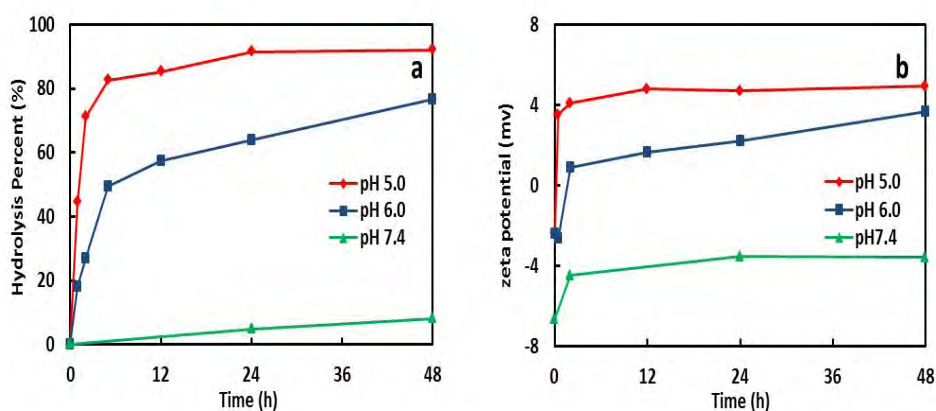


Figure 4. (a) The hydrolytic kinetics curves of the PCL-polyhis-S at different pH as a function of time detected by NMR. (b) The ζ -potential of the PCL-polyhis-S nanoparticles as a function of time at different pH.

As a consequence of the amide hydrolysis and imidazolyl group regeneration, the zeta-potential of the nanoparticles gradually became positively charged (Figure 4b). At pH 7.4, the ζ -potentials of the nanoparticles were very stable at about -4 mV during the testing time period as a result of the ionization of the succinamic acid groups. The ζ -potentials of the nanoparticles quickly increased to positive at 4 mV at pH 6.0 and 5.0 due to the hydrolysis of the amide and the protonation of the resulting imidazolyl groups. These results indicate that the PCL-Polyhis^S nanoparticles were negatively charged at the physiological pH and thus suitable for *in vivo* applications but could regenerate the Polyhis block at the acidic pH conditions.

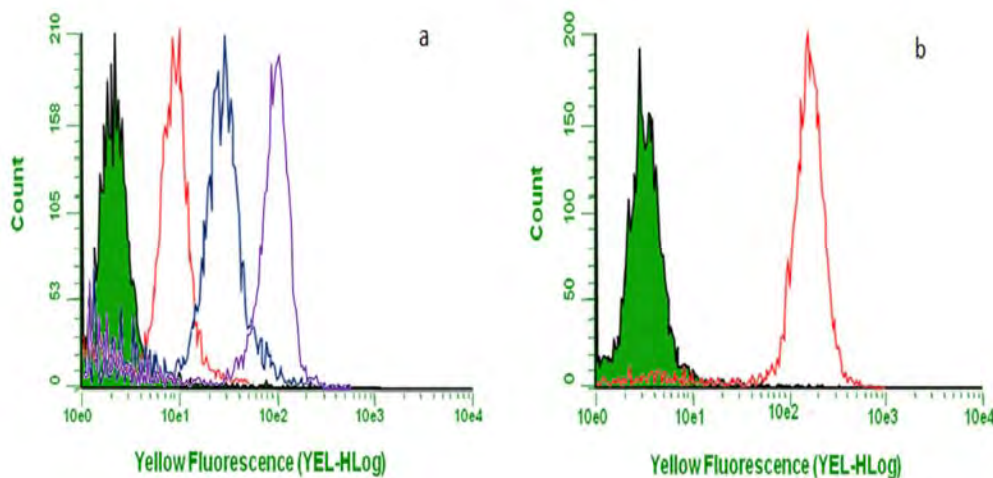


Figure 5. Cellular internalization measured by flow cytometer of (a) SKOV-3 cells with DOX loaded PCL-Polyhis^S nanoparticles for 1h (red curve, 23.2%), 5 h (blue curve, 56.6%), 12 h (purple curve, 82.6%) and control (green peak, 0.8%); (b) Folate free SKOV-3 cells with DOX loaded PCL-Polyhis^S (with 10% of folic acid group) nanoparticles for 1h (red curve, 91.2%) and control (green peak, 1.1%).

2.4. The cellular uptake of NPs

The cellular uptake of PCL-Polyhis^S nanoparticles was measured with flow cytometer (Figure 5). DOX was used as the fluorescence dye at the concentration of 1 $\mu\text{g/mL}$. After 1h, 5h and 12h incubations with SKOV-3 cells (Figure 8a), 23.2%, 56.6% or 82.6% of cells took up the nanoparticles, respectively. To accelerate the cellular uptake, folic acid (FA) group was introduced to the nanoparticles. FA is known to bind its receptors overexpressed on the cell membrane of many cancer types including breast cancer, and trigger receptor-mediated endocytosis.⁶⁻⁸ FA-PEG-PCL was mixed with the PCL-Polyhis^S copolymer at the mole ratio of 10% and then formed nanoparticles using the abovementioned method. SKOV-3 ovarian cancer cells overexpress folate receptors were used for the cellular uptake. As shown in Figure 5b, the cellular uptake was significantly enhanced (91.2%, 1h incubation), which indicates that the FA group induced the endocytosis.

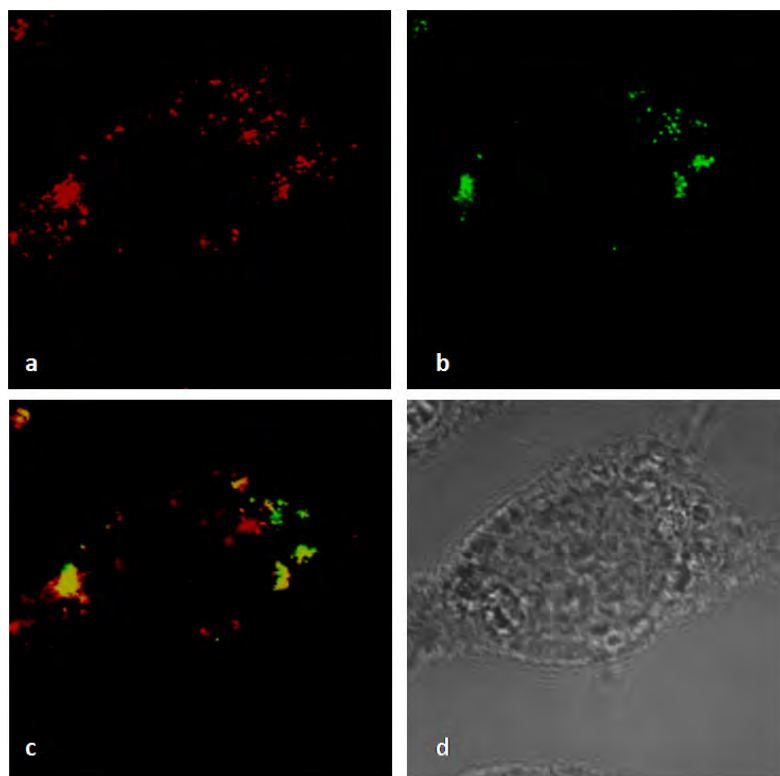


Figure 6. Subcellular localization of PCL-Polyhis^{-S}/nile red observed by confocal scanning laser fluorescent microscopy. Nile red channel (a); LysoTracker green (DND-26) channel (b); overlap of a and b channel (c); transmittance channel (d). Original magnification is 63 ×; 5 h incubation at 37 °C with of PCL-Polyhis^{-S}/nile red at nile red dose of 1 µg/mL.

The intracellular drug delivery and subcellular distribution of PCL-Polyhis^{-S} nanoparticles were subsequently studied using a subcellular compartment labeling method. The nanoparticles were loaded with nile red. The nanoparticles were then cultured with Skov-3 cancer cells for 5 and 12 h. LysoTracker green was used as the dye to label the lysosomes. As shown in Figure 6, many nanoparticles were internalized. Some of them were localized in lysosomes (yellow spots in Figure 6c). The others were not associated with lysosomes (red spots in Figure 6c), which might indicate that the nanoparticles already escaped from lysosomes with 5 h incubation.

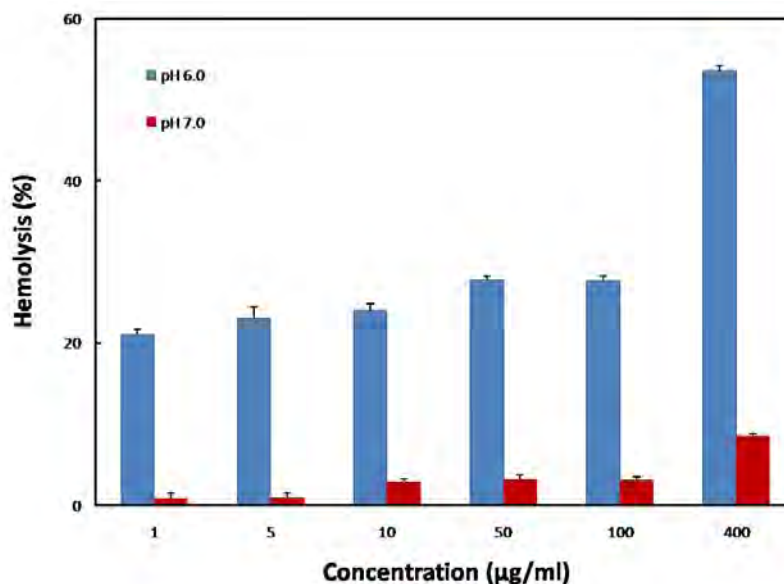


Figure 7. The hemolytic activity of PCL-Polyhis^{-S} nanoparticles on RBCs at pH 6.0 and pH 7.4 as a function of concentration (1 h incubation at 37 °C).

The ability of the PCL-Polyhis^{-S} nanoparticles escaping from lysosomes was further tested using a hemolysis assay, a measure of a drug carrier's rupture ability to lyse lysosomes.⁹ Figure 7 shows that at pH 7.0 PCL-Polyhis^{-S} nanoparticles only lyzed around 5% of the RBCs even at the concentration of 400 µg/mL, but at pH 6.0 could lyzed more than 20% RBCs at the concentration as low as 1 µg/mL. These results were consistent with those showed in Figure 6. The regenerated Polyhis of the nanoparticles at pH 6.0, ruptured the lysosomes efficiently. At pH 7.0, the nanoparticles concentration could not lyse RBCs even at 100 µg/mL and thus the PCL-Polyhis^{-S} nanoparticles were suitable for the *in vivo* application.

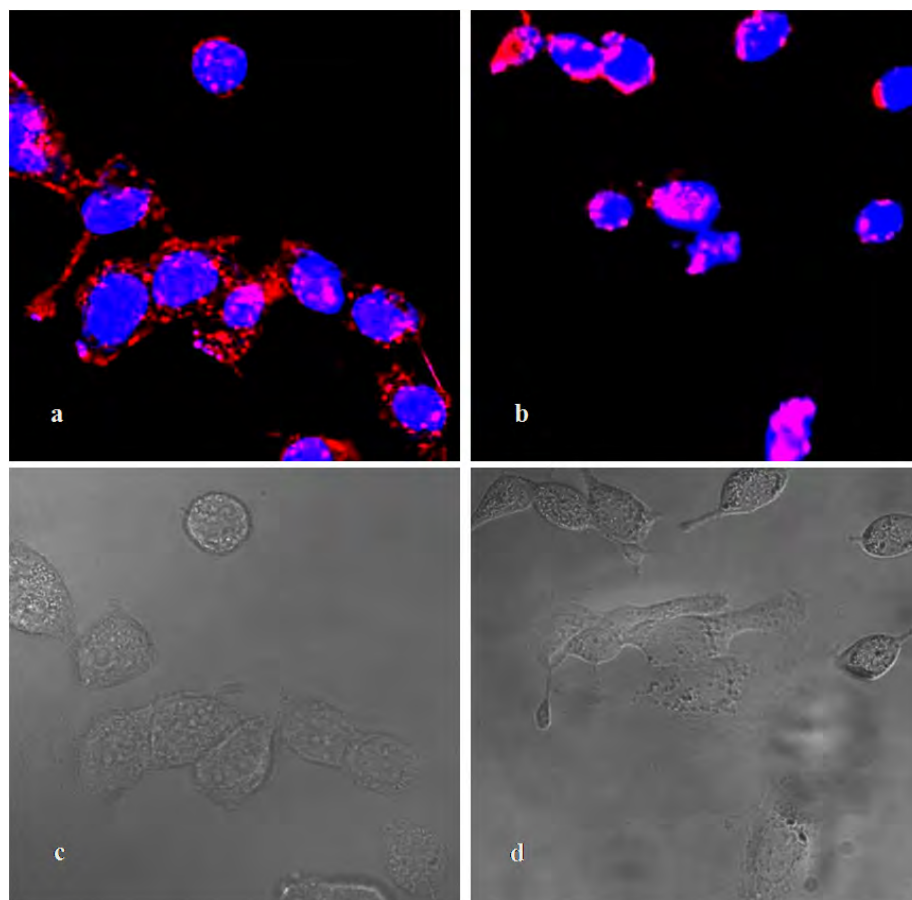


Figure 8. Nuclear localization of PCL-Polyhis^S/nile red observed by confocal scanning laser microscopy after cultured with SKOV-3 cells for 5 h. (a) or 12 h (b) at 37 °C. The nuclei were stained with DRAQ5 (blue). Nile red were assigned to red. Pink spots are PCL-Polyhis^S/nile red localized in the nuclei. Original magnification is 63 X. (c) is the transmittance channel of (a); (d) is the transmittance channel of (b).

2.5. The intracellular distribution of the NPs

The intracellular distribution of the PCL-Polyhis^S nanoparticles after long culture with cancer cells was observed by confocal laser scanning microscopy (Figure 8). SKOV-3 ovarian cancer cells were cultured with PCL-Polyhis^S/nile red for 5 h or 12 h before examination. Nile red was used as a dye to label the nanoparticles at the dose of 1 µg/mL. DRAQ5 was used as a dye to label the nuclei. After 5h culture, most PCL-Polyhis^S/nile red nanoparticles were outside of the nuclei, possibly located in lysosomes (Figure 6), but some were associated with or close to the nuclear membrane (red spots), and some even located in the nuclei (pink spots) (Figure 8a). After 12h culture, most of the nanoparticles located in the nuclei (pink spots, Figure 8b) and only few of them could be found in the cytoplasm. This is also agreeable with the nanoparticles' exceptional lysosomal lysis

capability shown in Figure 6. Therefore, the PCL-Phis^S nanoparticles regenerated as PCL-Polyhis and subsequently escaped from the lysosomes, and finally translocated.

2.6. In vitro Cytotoxicity

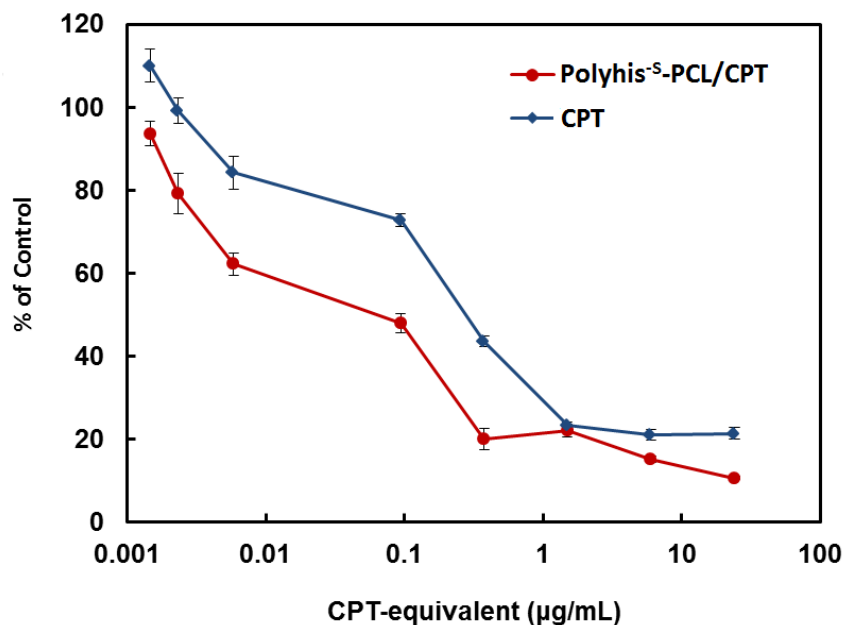


Figure 9. The cytotoxicity of CPT and PCL-Polyhis^S/CPT to SKOV-3 ovarian cancer cells as a function of CPT dose.

The cytotoxicity to SKOV-3 ovarian cancer cell line and DOX resistance MCF-7 cancer cell line of CPT or DOX loaded in FA-PCL-Polyhis^S was evaluated and compared with free CPT using MTT assays (Figure 9). FA-PCL-Polyhis^S/CPT showed a higher cytotoxicity than free CPT at most testing doses. The IC₅₀ of the free CPT was about 0.5μg/mL, while it decreased to 0.1μg/mL once encapsulated in the FA-PCL-Polyhis^S nanoparticles.

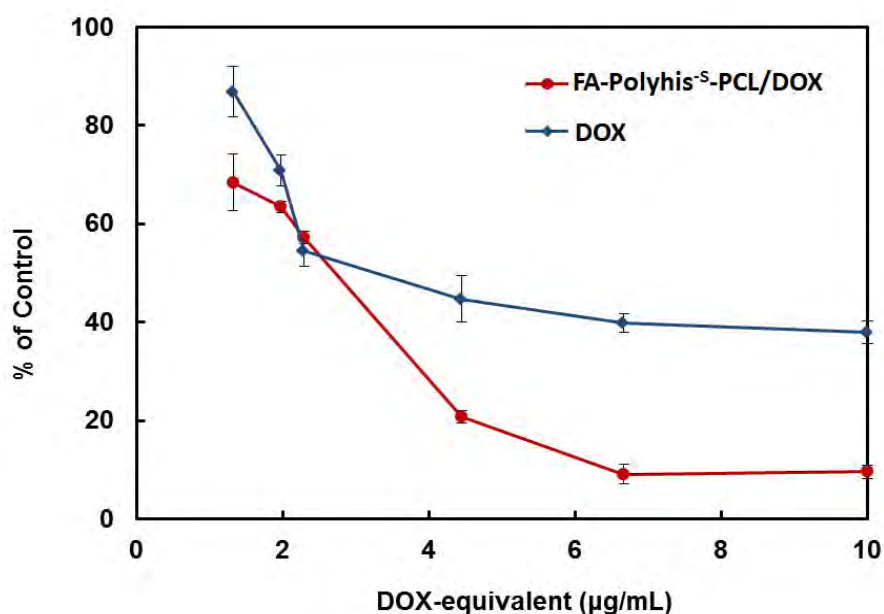


Figure 10. The cytotoxicity of DOX and FA-PCL-Polyhis^S/DOX to MCF-7/ADR DOX resistance breast cancer cells with 24 h treatments and 24 h post treatments.

As shown in Figure 10, FA-PCL-Polyhis^S/DOX showed higher cytotoxicity than the free DOX in most of the doses to MCF-7/ADR DOX resistance breast cancer cells. Free DOX had almost no dose-dependent cytotoxicity at doses higher than 2 ug/ml and more than 40% cells still survived even at 10 μg/ml dose. In contrast, DOX in the FA-PCL-Polyhis^S nanoparticles quickly reduced cell survival and less than 10% cell survived at 6.8 μg/ml dose. These results further indicate that the nanoparticles could be taken up by the cells efficiently and then delivered the drugs into the nuclei, leading to higher cytotoxicity.

3. Conclusion

We demonstrate a new pH-sensitive PCL block N-succinamicacidimidazole Polyhistidine (PCL-Polyhis^S) copolymer to be useful for *in vivo* nuclear drug delivery. The imidazole residues of the Polyhistidine are modified with succinamic acid group, which significantly enhances the water solubility of the Polyhistidine at the neutral pH and makes the nanoparticles negatively charged at neutral pH as well. Once the PCL-Polyhis^S nanoparticles are transferred to the tumor tissue or the cell lysosomes, the succinamic acid groups hydrolyze to regenerate the imidazole residues and thus the lysosomal-escaping ability of the polyhistidine is regained. By

functionalizing the PCL-Polyhis^S nanoparticles with FA-targeting moieties, the loaded drug (DOX or CPT) can be efficiently shipped to the cell nucleus to ensure high cytotoxicity.

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Key Research Accomplishments

1. We synthesized more efficient breast cancer cell-targeted charge-reversal drug-loaded nanoparticles for nuclear drug delivery to enhance the drug's cytotoxicity. It is ready for in vivo tests.

Reportable Outcomes:

Publications

1. Zhou Z, Shen Y, Radosz M, Murdoch WJ et al, pH-Responsive Polyhistidine Nanoparticles for Nuclear Targeted Drug Delivery, Manuscript to be submitted